PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 15/12, 15/13, 5/10 C12N 1/21, C12Q 1/68 A61K 37/02, 39/395	A1	(11) International Publication Number: WO 94/09131
		(43) International Publication Date: 28 April 1994 (28.04.94)

(21) International Application Number: PCT/GB93/02133

(22) International Filing Date: 15 October 1993 (15.10.93)

(30) Priority data:
9221657.1 15 October 1992 (15.10.92) GB

(71) Applicant *(for all designated States except US)*: SCOTGEN LIMITED [GB/GB]; Kettock Lodge, Campus 2, Aberdeen Science and Technology Park, Balgonie Drive, Bridge of Don, Aberdeen AB22 8GU (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : HARRIS, William, Joseph [GB/GB]; 18 Queen Street, Carnoustie, Angus DD7 7AB (GB). EMERY, Stephen, Charles [GB/GB]; 21 Cromlet Place, Oldmeldrum, Aberdeenshire AB51 0DW (GB). WINTER, Gregory, Paul [GB/GB]; 64 Cavendish Avenue, Cambridge CB1 4UT (GB). CARR, Francis, Joseph [GB/GB]; Birchlea, The Holdings, Balmedie, Aberdeenshire AB4 0XU (GB). MCGREGOR, Duncan, Patrick [GB/GB]; Cottage No. 2, Balcairn, Nr Oldmeldrum, Aberdeenshire AB51 0NE (GB).

(74) Agent: ARMITAGE, Ian, M.; Mewburn Ellis, 2 Cursitor Street, London EC4A 1BQ (GB).

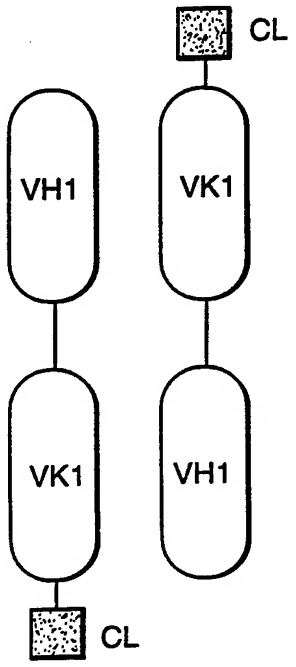
(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: RECOMBINANT SPECIFIC BINDING PROTEIN

(57) Abstract

A specific binding protein having first and second binding regions, e.g. antibody Fv fragments, which specifically recognise and bind to target entities, said binding regions being contained at least in part on respectively first and second polypeptide chains, said chains additionally incorporating respectively first and second associating domains, e.g. antibody V_H and V_L domains, which are capable of binding to each other, causing the first and second polypeptide chains to combine, thereby providing a single protein incorporating the binding specificities of said first and second binding regions. The first and second binding regions may recognise different target entities, giving a bispecific binding protein. Preferably the associating domains are derived from a human protein (i.e. one which has been exposed to the human immune system), so that the protein is less likely to provoke the human immune system when administered therapeutically. The binding protein is suitably produced by recombinant DNA expression.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**FR**	France	**MR**	Mauritania
AU	Australia	**GA**	Gabon	**MW**	Malawi
BB	Barbados	**GB**	United Kingdom	**NE**	Niger
BE	Belgium	**GN**	Guinea	**NL**	Netherlands
BF	Burkina Faso	**GR**	Greece	**NO**	Norway
BG	Bulgaria	**HU**	Hungary	**NZ**	New Zealand
BJ	Benin	**IE**	Ireland	**PL**	Poland
BR	Brazil	**IT**	Italy	**PT**	Portugal
BY	Belarus	**JP**	Japan	**RO**	Romania
CA	Canada	**KP**	Democratic People's Republic of Korea	**RU**	Russian Federation
CF	Central African Republic			**SD**	Sudan
CG	Congo	**KR**	Republic of Korea	**SE**	Sweden
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovak Republic
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LU**	Luxembourg	**TD**	Chad
CS	Czechoslovakia	**LV**	Latvia	**TG**	Togo
CZ	Czech Republic	**MC**	Monaco	**UA**	Ukraine
DE	Germany	**MG**	Madagascar	**US**	United States of America
DK	Denmark	**ML**	Mali	**UZ**	Uzbekistan
ES	Spain	**MN**	Mongolia	**VN**	Viet Nam
FI	Finland				

RECOMBINANT SPECIFIC BINDING PROTEIN

This invention relates to recombinant bispecific (heterodimeric) and/or monodimeric bivalent specific binding proteins, for example antibodies.

Since the development, in 1975, of monoclonal antibody hybridoma technology (ref 1) it has been possible to produce a large number of specific antibodies which have been used widely as research, therapeutic and industrial tools. However, general experience indicates that murine monoclonal antibodies display reduced therapeutic efficacy which may be attributed to elicited immunoglobulin responses by the human immune system, or alternatively poor initiation of defence mechanisms such as complement activation or the recruitment of human effector cells or have inappropriate pharmcokinetics in vivo.

Approaches to circumvent these limitations have been achieved by the use of humanised monoclonal antibodies (ref 2), antibody fragments (refs 3 and 4) and "bispecific" antibodies (monovalent bispecific reagents)(refs 5 and 6). Bispecific antibodies may be described as recombinant antibodies capable of binding two different antigenic sites and thus contain antigen binding domains derived from two different sources, and which are brought into association by complementary interactive domains within the antibody molecule.

Bivalent antibodies of a monospecific nature may be derived from hybridomas and similarly, bispecifics by the

fusion of two hybridoma lines expressing antibodies with different specificities. However, using this strategy, the application of bispecifics has been limited by the difficulty in efficiently producing and purifying such molecules and additionally, the effector functions intrinsic to complete antibody molecules (such Fc receptor and complement binding) have led to undesirable interactions.

Recombinant DNA technology coupled with the recent advances in the fields of monoclonal antibody and protein engineering has enabled access to large selection antibodies and antibody fragments which have monospecific recognition and binding properties for a wide variety of different antigens. The lack of effector functions and the ability of immunoglobulin Fab, F_v and single chain F_v (scF_v) fragments to penetrate effectively into tissue from the vascular system has made these molecules excellent candidates for drug delivery systems and imaging tools (ref 7). However, unlike complete antibodies, these fragments are monovalent, each carrying a single antigen binding site

Dimerisation techniques which promote the formation of bivalent complexes (or even the formation of multivalent complexes) may therefore be of great importance when one considers constructs, for example F_v or Fab fragments, in which the monovalency of the product precludes polyvalent binding to the antigen, providing an avidity factor.

In addition, most bacterially produced F_v, Fab and even scF_v fragments have shown lower avidities than their

progenitor monoclonal antibodies (refs 8 and 9). Since there is no extra stabilization provided by the association of other domains, as in the case of a complete antibody, the stability of F_v's is substantially dependant on heavy and light chain variable domain association. Since the heavy and light chain domain association stability is dependant on the residues displayed at the domain interface (which is partly formed by residues belonging to the CDR regions) the dissociation constants for Fv and similiar constructs may vary greatly from antibody to antibody. Thus antibodies with poorly associating variable regions are likely to show lower binding affinities, relative to their parent molecules, when in a small fragment configuration.

Therefore there are good reasons to develop methodologies for the production of dimeric antibodies which may allow one to increase the binding affinity of a particular antibody via bivalent binding and/or additional stability due to further domain association.

In addition to the production of bivalent antibodies, a major application of molecular dimerisation lies in the production of "bispecific" or "bifunctional" antibodies in which the association is by definition "heterodimeric" (each subunit containing different V domains).

In vitro experiments have shown that bispecific antibodies can be effective in cross-linking cytotoxic effector cells to target cells and stimulating the cytotoxic destruction mechanism. Similarly, bispecific antibodies may be utilized to associate and bring into

close proximity certain molecules eg. a drug or a toxin, a radiolabelled hapten or inactivating protein to other entities, such as a particular protein or cell type, via the specific recognition and binding of the individual binding domains. In addition, such "bifunctional" antibodies may also be used to develop novel immunoassays and/or diagnostic assays. Since the bispecific antibodies are capable of binding to two distinct antigens, they may be designed to bind enzymes, therapeutic agents, radiolabels or cells to a target without the need for chemical modification. Hence bispecific entities are likely to demonstrate more efficient and specific cell killing than antibodies directly conjugated to cytotoxic agents and furthermore avoid any of the adverse immune responses which may be elicited by chemically modified antibodies.

Bispecific antibodies are likely also to prove useful for accelerating and promoting the association/dimerization of other molecules which may have themselves signalling, effector or reporter functions when in a associated/dimeric state. Therefore, bifunctional antibodies may also have an important role as "molecular switches" as well as "cross-linkers" together with a multitude of other uses (both diagnostic or therapeutic) to which a dual binding specificity may be applicable.

Although the possible roles for which bispecific antibodies may be advantageous are numerous, the clinical applications and other uses for bispecific antibodies have

been limited by the difficulty in efficiently producing and purifying such molecules.

Methods used to assemble bispecific or other dimeric antibodies include chemical cross-linking, disulphide exchange or the utilization of hybrid-hybridomas or heterotransfectomas. These methods have proved as yet unsatisfactory due to the purity of the products required for successful construction, the production of heterogeneous and ill defined products and especially in the case of Fab fragments the low affinity of the partners (Fd, V_LC_L) for each other.

To address these problems a recent development for the production of heterodimeric $F(ab)_2$ molecules has employed the C-terminal fusion of peptide sequences corresponding to the leucine zipper regions of the Fos and Jun transcription factors (Kostelny et al., J. Immunol. 148, 1547-1553, 1992)(ref10). These sequences preferentially form heterodimers and therefore are effective in promoting bispecific formation when fused to two different Fab fragments.

To achieve bivalency (i.e. two binding sites with the same specific within one antibody based entity), Pack and Pluckthun (1992)(ref 11) designed mini-antibodies based upon single chain F_v fragments with a flexible hinge region and an amphiphilic helix fused to the C-terminus of the antibody fragments. They have shown that such molecules have antigen binding characteristics nearly identical to whole antibodies. A different approach has been described

by Carter et al., (1992)(ref12) based upon the expression of Fab in E. coli and subsequent chemical linking of free thiols to generate $(Fab')_2$.

According to one aspect of the present invention there is provided a specific binding protein having first and second binding regions which specifically recognise and bind to target entities, said binding regions being contained at least in part on respectively first and second polypeptide chains, said chains additionally incorporating respectively first and second associating domains which are capable of binding to each other, causing the first and second polypeptide chains to combine, thereby providing a single protein incorporating the binding specificities of said first and second binding regions.

Preferably, said first and second binding regions recognise different target entities.

Suitably said associating domains are derived from an antibody (eg IgA, IgD, IgE, IgG, IgM), and much of the description which follows uses antibody domains by way of illustration. However, they could be derived from other molecules which are capable of associating, such as natural ligand/receptor combinations or binding regions of natural dimeric proteins. Preferably the associating domains are derived from a human protein (ie one which has been exposed to the human immune system).

Said associating domains may be two identical domains which are capable of association, even if they do not normally associate in nature. Conveniently said

associating domains are selected from:

antibody V_H and V_L regions,

antibody C_H1 and C_L regions,

antibody $V_H.C_H1$ and $V_L.C_L$ regions.

Preferably the first and second binding regions are antibody antigen-binding domains, eg comprising V_H and V_L regions contained in a Fab fragment or in a single-chain Fv fragment.

However, either or both of the binding domains could be derived from just one of the V_H or V_L regions of an antibody, most suitably from the V_H region of an antibody (ref 13).

The invention also includes DNA encoding the polypeptides of such a specific binding protein; optionally contained in one or more expression vectors; host cells containing such recombinant DNA and capable of expression of the DNA to produce the polypeptides of the specific binding protein.

The invention also provides a process which comprises expressing the recombinant DNA in such a host cell to produce the polypeptides encoded thereby, and where necessary performing post-translation manipulation or processing of the polypeptides, to produce the specific binding protein.

The invention further provides a process for producing a specific binding protein as set forth above, which comprises:

(i) cloning or synthesising DNA encoding said first

and second binding regions,

(ii) joining in reading frame to the DNA of said first and second binding regions DNA encoding respectively said first and second associating domains,

(iii) providing one or more expression vectors containing said fused DNA constructs of (i) and (ii),

(iv) inserting the expression vectors into a host organism, (v) culturing the host organism to express the encoded polypeptides, and

(vi) causing or allowing said first and second associating domains to combine to form the specific binding protein.

Thus, in general terms the invention provides recombinant bispecific (heterodimeric) and/or monodimeric bivalent specific binding proteins, for example antibodies, in which the specific association of the component modules is accomplished by using the recognition and natural homo- or hetero-dimerization of additionally fused associating domains.

Methods for the production of the aforementioned bispecifics and/or dimerics (and variants thereof) are also disclosed.

Unless the context requires otherwise, the terms "antibody" and "antibody fragments" are hereinafter used synonymously. Similarly the terms "antibody" and "immunoglobulin" are to be treated in a likewise manner.

The accompanying drawings show diagrammatically:

Fig 1 a general scheme for bivalent and bispecific

antibody formation,

Fig 2 an experimental example for bivalent recombinant antibody formation,

Fig 3 an example of minimal configuration bispecific recombinant antibody formation, and

Figs 4 to 9 examples of bispecific recombinant antibody formation.

Fig 10 shows the arrangement of genes in the various vectors used for antibody fragment expression in E.coli. All coding regions were cloned downstream from a lac promotor in pUC 19. VH and VK and CH and CK are the antibody fragment variable (V) and constant (C) domains respectively, Pb denotes a Pel B leader sequence and the Tag gene encodes a peptide for antibody fragment detection with a secondary antibody. The Tag gene product was not used in these studies. The linker and termination signal were engineered into the vectors as described in the materials and methods. pSW1-Fab/Pa is identical to the pSW1-VHD1.3-VKD1.3-TAG1 vector described by Ward et al. (1989) with the exception that the VH and HK domains encode anti-P. aeruginosa rather than anti-lysozyme binding domains. pDM1 is a derivative of pSW1-Fab/Pa.

Fig 11 shows denaturing SDS-PAGE of affinity purified anti-P. aeruginosa Fab (lane 2) and ScF_v* (lane 3). 2-5 mg of each sample were electrophoresed through a standard denaturing 10% SDS-PAGE gel.

Fig 12 show the relative antigen binding capacities of affinity purified anti-P. aeruginosa Fab (■) ScF_v*(●) and

the original chimeric antibody (o).

Fig 13 shows a Western blot showing the cross-reactivity of the anti-P. aeruginosa with a number of Gram-ve bacteria.

Fig 14 shows the different conformational forms of anti-P. aeruginosa ScF_v* and Fab. (a) A 7.5% non-denatruring polyacrylamide gel showing multimeric forms (I-III) of ScFv* (lanes 1-3) and a single monomeric form of Fab (lane 4). HPLC purification of monomeric (III), dimeric (II) and trimeric (I) forms of ScF_v*.

Fig 15 shows a comparison of the ability of HPLC purified monomeric ScF_v* (■), dimeric ScF_v* (□) and the original chimeric antibody (o) to bind P. aeruginosa.

The advantage of the present invention, for the association of the bispecific heterodimer or simple homodimeric bivalent formation, over existing techniques lies in the given nature of the fused domains utilized for this purpose. One of the major disadvantages of chemical modification or the Jun/Fos type approaches concerns their potential to elicit an immune response against "foreign" chemical constituents and peptide sequences when used in human therapy. The technique of using antibody domains to achieve dimerization of antibody constituents avoids this problem by using natural antibody domains which should not induce an immune response in humans especially where the antibody domains are reshaped (humanised) or derived from natural human antibodies. Such dimeric antibodies ought to closely approach the immunogenic characteristics of a

natural human antibody and thus should have an extended half-life in circulation compared to bispecifics prepared by other means as discussed previously.

In addition to promoting dimerisation through specific intermolecular recognition, the use of fused C-terminal domains (with known dimerisation properties) is likely to lead to stabilization of the dimeric constructs produced due at least to the increase in area of hydrophobic interface interaction. For example the association of heavy and light chains (K_A >10^{10} M^{-1}) is derived from the combination of relatively weak V_H-V_L (K_A~10^6 M^{-1}) and C_H1-C_L (K_A~10^7 M^{-1}) interactions, indicating that the association of the two domain pairs is at least additive (ref 14).

The present invention relates to the development of bivalent and other dimeric antibodies especially for use in therapy or for in vivo diagnosis. The successful targetting of antibody molecules to sites of disease upon repeated administrations is dependant on these antibodies provoking little or no immune reaction. Maximisation of the natural human antibody sequence content of the bispecific antibodies will prospectively enhance the perspective for long-term repeat administration of these antibodies, for example in the treatment of chronic disease. Notwithstanding this, other applications as yet undetermined or not described herein for which mono-, bi-, or multi-specific antibodies/antibody fragments (produced via the means of association described in this invention) may be applied will be also encompassed by this patent.

The general concept of association of specific binding chains which is applicable to the present invention may be illustrated by reference to figure 1. In this diagram, the entity has been divided into four components A, B, C and D in order to simplify discussion of the inventive process.

As previously discussed, a wide variety of antibodies (and fragments thereof) are readily available. Thus, there are numerous combinations of constructs which may be produced within the parameters of this invention. One may consider the specific specific binding regions or the associating domains as derived variously from such entities as Fab, F_v, scF_v, F_d, V_L/C_L, Fc or F(ab)' fragments, or individual domains such as C_H3, C_H2, C_H1, C_L, V_H or V_L (or even various combinations of these domains).

The specific binding regions and the associating domains are fused to each other either directly or via peptide linkers of different lengths (the linker sequence composition and length, being tailored to the particular antibody configuration and foreseen application). The components B and D may be nominally seen as additionally fused domains, although in certain configurations they may themselves be part(s) of the same antibody from which A and C are derived. The fragments or domains may be derived from one or more sources depending again on the chosen format and foreseen application of the particular construction. A further augmentation, also foreseen in this invention, is that for particular molecular configurations and destined applications it may be

advantageous to introduce or use existing cysteine residues to create inter-domain disulphide bridges in order to further stabilize the constructs. The reduction to produce these covalent bonds may be performed in vivo or in vitro depending on the construct and relative merits of this additional process.

It will be apparent to those skilled in the art that the choice of any particular type of antibody or antibody fragment is not fundamental to the inventive concept described here. Similarly any immunogobulin which provides the desired specificity may be employed. An extension of the invention also foresees the use of any natural molecules (or fragments thereof) which form specific associations (such as cell surface receptors and their ligands) which have been exposed to the immune system and show little or no immunogenicity within the said system.

A valuable embodiment of this invention foresees the dimerization of the constituent partners via the homodimeric association of additionally fused antibody domains (or other immuno-silent molecules). Such a strategy may be used to produce dimeric antibodies. This embodiment is expected to be more suited towards the dimerisation of single antibody species to form homodimeric, bivalent antibodies, since is recognised that a mix of product assemblies may arise when this technique is used for the co-expression and association of two different antibody species to form a heterodimeric, bispecific antibody.

The following specific description illustrates how the invention may be carried out. The "Reference example" provides an illustration of the basic technology by which specific binding and associating fragments can be fused and caused to associate, while Examples 1 and 2 which follow provide specific embodiments of the invention to whose manner of execution can draw on the technology of the Reference example.

Reference Example

The concept of using dimeric association of fused domains to enable formation of a bivalent species may be simply illustrated by the following example designed for production of a bivalent homodimeric $(scF_v)_2$ antibody. With reference to figure 1, in this embodiment, the components A and D are V_H and V_L domains respectively fused via a linker to a cognate V_L and V_H domain (components B and C respectively). This format for the production homodimeric bivalent antibodies has been experimentaly tested using a simple model system (see Fig. 2) in which V_H and V_L domains of an anti-Pseudomonas aeruginosa antibody were fused via a linker peptide. This construct, when expressed in Escherichia coli and subsequently purified, was found to exist in monomeric, dimeric and multimeric forms as analysed by HPLC and non-denaturing PAGE. The dimeric form was predominant, and was found to have an antigen binding profile similar to the parent antibody, furthermore, it was found that the bivalent form was produced spontaneously.

Experimental

For this study the pUC based pSW1-VHD1.3-VKD1.3-TAG1 expression vector (Ward et al., 1989)(ref13), encoding anti-P. aeruginosa (PSV) antibody heavy and light chain variable region genes was employed. A novel single chain antibody construct was generated a fourteen amino acid linker described by Chaudhary et al., (1990)(ref 15) by modification of the original Fab encoding vector. This novel scF_v* construct possesses a human kappa chain domain fused to the 3' end of the VK domain for detection purposes. Results indicated that when expressed, this scF_v* construct formed multivalent products spontaneously.

Bacterial strain

All vectors were transformed and expressed in the E. coli strain XL-1-Blue (supE44 hsdR17 recA1 endA1 gyrA46 the relA1 lacF' [proAB+ lac19 lacZ M15 Tn10 (tet^r)]. The tetacycline selectable F' pilus allows strict control of expression of pUC based vectors.

Vectors construction

The PSV scF_v* expression vector was constructed form a pSW1-VHD1.3-VKD1.3-TAG1 vector derivative (pSW1-Fab/Pa) using the polymerase chain reaction (PCR) mutagenesis method of Higuchi (1989)(ref16). The linker was incorporated using a two stage procedure. Two separate amplifications were carried with the primers

VHBAK:5'-AGGT(C/G)(C/A)A(G/A)CTGCAG(G/C)AGTC(T/A)GG

with

LINKFOR:5'-CGATGTCATCCACTTTAGATTCAGAGCCAGAGCCAGAAGATT TGCCTTCTGAGGAGACGG

and

VKFOR:5'-GTTTGATCTCGAGCTTGGTGCC

with

LINKBAK:5'-GTCACCGTCTCCTCAGAAGGCAAATCTTCTGGCTCTGGCTC TGAATCTAAAGTGGATGACATCGAGCTG.

The vector pSW1-VHD1.3-VKD1.3-TAG1, expressing an anti-P.aeruginosa (**pSW1-Fab/Pa**), was used as template DNA.

The PCR contained 2 units of Taq polymerase (BCL, Lewes, East Sussex, England), 1.25mM dNTPs, 1mM of each either VHBAK and LINKFOR or VHFOR and LINKBAK, 50 ng pSW1-Fab/Pa and 5µl 10x reaction buffer (BCL) made up to 50µl with de-ionised water. All reactions were UV treated for ten minutes prior to the addition of polymerase and template, and then overlaid with mineral oil. PCR was performed using a Techne PHC-2 thermal cycler (Scotlab, Aberdeen, Scotland) using the following parameters: 94°C, 1 minute, 60°C, 1 minute, 72°C, 1 minute for 30 cycles. The purified products of these reactions were mixed and seven cycles of PCR were performed without primers (94°C, 1 minute; 72°C, 1 minute). This reaction was then maintained at 94°C while VHBACK and VKFOR primers were added. A further 30 cycles of PCR were then performed (94°C, 1 minute; 65°C, 1 minute; 72°C, 1 minute). The reaction products were electrophoresed through a 2% (w/v) agarose-

TAW gel and the 650 bp product purified into 10µl of de-ionised water by the Prep-a-gene procedure (Bio-Rad Ltd, Hemel Hempsted, Herts., England). The product was digested with PstI and XhoI then ligasted into PstI/XhoI digested pSW1-Fab/Pa under standard conditions to construct the pDM1 vector (fig 10).

Antibody fragment expression

The expression vectors pDM1 encoding the anti-P. aeruginosa scF_v* antibody fragment and pSW1-Fab/Pa encoding the equivalent Fab fragment (each with the fused light chain constant region domain for detection purposes) were assessed. Expression conditions were modified from Ward et al, (1989)(ref 13): XL1-blue transformed with one of the above vectors was grown in 5ml 2xTY cultures containing 1% glucose, 100µg/ml ampicillin and 12.5µg/ml tetracycline at 37°C, overnight. Ten microlitres of overnight culture was then used to inoculate fresh medium for expression of antibody fragments. Expression was carried out at 26°C for six to eighteen hours in Terrific broth or 2xTY containing 0.1mM IPTG, 100µg/ml ampicillin and 12.5µg/ml tetracycline. Antibody fragments were detected in culture supernatants by ELISA or could be purified directly from cell pellets as described below.

Affinity purification of antibody fragments

Anti-P. aeruginosa scF_v* and Fab fragments were

purified by affinity chromatography. Five hundred millilitre cultures of bacteria producing either anti-P. aeruginosa Fab or scF_v* were centrifuged at 4000 rpm for 20 minutes. The supernatant was discarded and the cell pellet sonicated for 1 minute in 10ml of PBS to release periplasmic antibody fragments. This solution was concentrated to a 1ml volume in a Centricon-10 column (Amicon, Stonehouse, Glos, England) and loaded onto an anti-human FAb affinity column (Pierce, Warriner, Cheshire England) prepared according to the manufactures instructions. Seven 1ml fractions were eluted in 0.1M glycine buffer pH2.8. The fractions were dialysed overnight against PBS and analyzed by polyacrylamide gel electrophoresis.

Detections of antibody fragments for sandwich ELISA

Bacterial supernatants and antibody fragments (FAb and scF_v*) purified from cell pellets were assayed in 96 well flat bottomed polystyrene plates (Nunc, Denmark). The plates were coated overnight at 4°C with 1µg affinity purified goat anti-human IgG Fab antibody (Sigma Chemical Co., Poole, Dorset, England) in 50µl phosphate buffered saline (PBS). The plates were blocked for 1 hour at 37°C with 200µl of 2% (w/v) BSA (fraction V, Sigma) in PBS and then washed twice with 200µl PBS. Fifty microlitres of culture supernatant or purified antibody fragments were added to each well and the plates incubated at 37°C for 1 hour. After this time the plates were washed four times

withe 200µl 0.05% (v/v) Tween 20 in PBS (Tw20/PBS). Fifty microlitres of horse radish peroxidase conjugated goat anti-human IgG Fab antibody (Sigma), diluted 1:2000 in Tw20/PBS was added to each well and incubated at 37°C for 1 hour. The plates were then washed six times in 200µl Tw20/PBS and the assay developed with 50µl 1mg/ml 0-phenylendiamine dilydrochloride (Sigma) and 1µl/ml 30% (v/v) hydrogen peroxide (Sigma) in 0.1M citrate-phosphate buffer pH 6.0. The reaction was stopped with 100µl 0.5M citric acid and the optical density at 450nm was read using a Bio-Rad M450 plate reader. Positive control anti-P pseudomonas mouse-human chimeric IgG_1 antibody was provided by Scotgen Ltd, Aberdeen. This antibody was derived from the original murine patent immunoglobulin from which the Fab and scFv* variable domains were obtained.

Detection of antigen binding of ELISA

This assay was performed essentially as the ELISA described above but with the following modifications. P. aeruginosa were grown overnight in Luria broth, pelleted an resuspended in PBS as a volume representing half of the volume of the original culture. The cells were then heat treated at 65°C for 10 minutes. 96-well polystyrene plates were coated overnight with 50µl of heat killed P. aeruginosa. After blocking the plates with Tw20/PBS and washing with PBS, 50µl of bacterial supernatant, purified antibody fragments or the mouse/human chimeric anti-P. aeruginosa antibody supernatant were added to each well and

incubated at 37°C for one hour. After the plates were washed four times with PBS the assay proceeded as described in the previous section.

Western blotting

The specificity of the scF_v* expressed was investigated by Western blotting. One million P. aeruginosa, P. fluorescens, Al. xylosidans, Ac. calcoaticus and O. anthropi were electorphoresed through a standard 10% SDS polyacrylamide gel with Rainbow size markers (Amersham Int., Aylesbury, Bucks, England). The gel was electorblotted at 0.8 mA/cm^2 in a Hoeffer T70 semi-dry blotter onto Immobilion-P membrane (Millipore Corp., Bedford, Mass, USA). The membranes were blocked overnight in 5% (w/v) milk powder in PBS. scF_v* (1µg/ml) was incubated for one hour in 0.1% (v/v) Tween 20 in PBS followed by three 10 minute washed in 1% (v/v) Tween 20 in PBS. The membrane was then incubated with a 1:5000 dilution of an anti-human kappa chain or anti-human Fab antibody conjugated to horse radish peroxidase in 5% (w/v) milk powder, 1% (v/v) Tween 20 in PBS for 30 minutes at 37°C. scF_v* binding was detected using the ECL chemiluminescent system (Amersham Int.).

Non-denaturing polyacrylamide gel electrophoresis

Affinity purified Fab and scF_v* were separated on a 7.5% non-denaturing polyacrylamide gel using the method of Ornstein (1964)(ref 17) to determine the conformation of

the molecules.

HPLC purification of scF_v* fractions

Forty microgram samples of scF_v* were analyzed using a Zorbax GF250 size exclusion column on a Gilson HPLC comprising a model 302 pump, an 802L manometric module, a 811 dynamic mixer, a 116 UV detector and a 201 fraction collector controlled by Gilson 714 software. The analysis was done using both PBS and 0.2M sodium phosphate buffer at a flow rate 1ml/minute. Eluate was monitored at 280nm, 01AUFS and 0.5 ml fractions were collected and analyzed by ELISA and non-denaturing polyacrylamide gel electrophoresis.

scF_v* and Fab purification and antigen binding

The presence of the light chain constant region allowed purification of both Fab and scF_v* directed against P. aeruginosa. Similar amounts (2-5mg) of both proteins were obtained when periplasmic cell extracts derived from 500 ml of IPTG induced overnight cultures were loaded onto affinity columns. The purity of the eluted proteins was assessed by denaturing SDS-PAGE with Coomassie Blue staining (fig 11).

The relative antigen binding capacities of affinity purified Fab (pSW1-FAb/Pa), scF_v* and chimeric antibody were compared. The chimeric antibody and the scF_v* gave similar binding profiles, indicating that the scF_v* had an affinity close to the original monoclonal antibody. Fab binding was

2-3 times weaker than either that of chimeric or scF_v* (Fig 12). Western blotting was employed to test the specificity of the scF_v* protein. One band of approximately 21 kd was observed when scF_v* was tested against P. aeruginosa antigens (Fig 13). Cross reactivity with Al. xylosidans and Ac. calcoaticus but not O. anthropi, F. mulitivoran or P. fluorescens was noted (Fig 13). These are all Gram negative bacteria.

Analysis of scF_v*

Differences in conformation of bacterially produced Fab and scF_v* molecules was thought to be responsible for the changed affinities observed in ELISA. Non-denaturing gel electrophoresis revealed that Fab (pSW1-Fab/Pa) was identifiable as a single unit while scF_v* was present as a series of multimeric forms (Fig 14a). Monomeric and dimeric forms of scF_v* appeared to predominate. HPLC purification by size exclusion allowed separation of the monomeric and dimeric scF_v* forms. Comparison of the elution times with those of molecular weight marker proteins confirmed the presence of both monomeric and dimeric forms of scF_v* (Fig 14b). An ELISA was employed to determine the ability of the monomeric and dimeric scF_v* fragments to bind P. aeruginosa and revealed that the dimeric form had affinity similar to the chimeric antibody while the monomeric form had a lower affinity (Fig 15).

Discussion

The bivalent component thus shows similar binding characteristics to the intact antibody while the monomeric fragment shows greatly reduced binding. Dimeric and monomeric forms can be easily separated by gel filtration, offering a simple method for preparing pure dimeric antibody.

The scF_v* dimers dissociate into monomers in non-reducing SDS gels and appear, therefore, not linked by disulphide bonds (data not shown). Our interpretation of the data is that the dimer formation of the $(scF_v*)_2$ is due to non covalent intermolecular V_H/V_L domain association; the association occurring between domains of different scF_v* polypeptides as indicated in figure 2. The resulting dimer is bivalent and shows a binding affinity to the chimeric antibody for the P. aeruginosa antigen.

Further Methods For Bispecific Recombinant Antibody Formation

The present invention discloses novel bispecific antibodies in which dimerization of the constituent partners is achieved via the heterodimeric association of additionally fused natural antibody domains. For example, antibodies may consist of two different Fab or scF_v antibody fragments (or any other antibody/antibody fragment combination) linked to either a V_H or V_L polypeptide by gene fusion. The desired bispecific antibody is therefore specifically associated by the recognition and natural heterodimerization of the fused V_H or V_L domains. The

present invention also discloses methods for the production of novel bispecific antibodies.

The simplest format envisaged for the production of a bispecific antibody would be the association of two single chain antibodies scF_v1 and scF_v2. If one relates to the general scheme shown in figure 1, the domains A and C would be V_H1 and V_L2 respectively, and domains B and D; V_L1 and V_H2 respectively (see Fig.3). In this miminal configuration it is clear that both the two domains of each individual peptide chains may associate to form normal scF_vs and additionally, that homo- and heterodimers (and possibly larger multimeric associations) of the two peptide chains may be produced by intermolecular domain association. A preferred embodiment of this invention is a bispecific antibody, formed by a C-terminal fusion of either a V_H or V_L antibody domain (component B) to one scF_v antibody fragment (component A) and its corresponding partner (component D) to the second scFv (component C). Such a species is illustrated in Figure 4.

Similarly for bispecific $F(ab)_2$ heterodimers, either the V_H or V_L (components B and D) could be added by C-terminal fusion to the V_H/C_H1 polypeptide chains of the Fab fragments (components A and C). Using this approach, Fab dimerization, to form the bispecific antibody, should be promoted by the spontaneous and specific association of the attached V_H and V_L domains. See Figure 5.

Examples of other alternative embodiments of this invention may involve the use of other naturally occuring

antibody domains which form heterogeneous (dimers such as $V_H.C_H1/V_L.C_L$ or C_H1/C_L) as C-terminal fusions (components B and D) which are illustrated in figure 6-9. This and other possible conformations have been alluded to previously in the text.

It will be apparent to those skilled in the art that the choice of any particular type of antibody or antibody fragment is not fundamental to the inventive concept but it is clear that the specificity and affinity of the C-terminal domains chosen will greatly influence the end product(s). Any domain(s) which provides the desired dimerization and recognition specificity required may be employed. However, it is recognised that, for example, specific V_H domains will associate more efficiently with certain V_L domains than others (K_D values for F_v's varying from 10^{-4} M - 10^{-7} M) (ref 18) and thus the V_H/V_L binding pair of a specific bifunctional should possibly be optimised to promote heterodimer formation and also to minimise homodimer formation (especially when producing bispecifics). Similiarly it is also foreseen that molecules may be modified in order to improve the specificity and/or binding affinity of the molecules for each other and thus improve their dimerisation properties (ref 19). This, for example, may be achieved via engineering, eg mutation or chemical modification, of the inter-domain hydrophobic contacts, introduction of a metal (or other molecule binding site) between the domains to stabilize association, introduction of strategic cysteine residues to form

disulphide bridges, etc.

If supplementary F_v domains are created when producing a bivalent or bispecific antibody (as may be the case when using the V_H/V_L domain combination as fusions), the antibody may, in fact, be trivalent. This extra antigen binding site may be useful itself. If the binding site recognizes a different type of antigen or ligand than the other sites, this property may be used for purification (ligand bound to matrix), stability (ligand added and bound to site thereby "cross-linking" the two domains of the F_v) or as a label (added ligand-reporter complex). This idea would also be valid for bispecifics, if one type of binding site is used for ligand binding it may be used for the same purposes as described above for the trimer.

The present invention provides an antibody with a minimum of two F_v domains each comprising of structural framework with the relevant CDRs which provide the antibody with the capability to bind specific molecules such as antigens. The invention may be exemplified ,but not limited, in a configuration in which the antigen recognition and binding domains (and any other affiliated sequence such as constant regions etc) are fused at the C-terminal end to "association" domains such as either a V_H or V_L antibody domain respectively. Association of the constituent partners is achieved via the natural heterodimerisation of the fused V_H and V_L antibody domains (see figs 4 and 5).

Processes for the production of such antibodies and

genes encoding such antibodies are also provided by the present invention.

Corresponding to a preferred process of the present invention, there is provided a method to produce bispecific $(scFv)_2.V_H/V_L$ or $F(ab)_2.V_H/V_L$ entities (see figs, 4 and 5) or derivatives of these with either C_H1/C_L or $V_H.C_H1/V_L.C_L$ fused domains (see figs.6-9). This method comprises the following basic scheme:

(i) Cloning or synthesis of the antibody F_v and/or Fab genes from the antibodies which exhibit the desired binding properties. The genes should be inserted into plasmid or phage vectors suitable for proliferation and /or gene expression.

(ii) The addition of the V_H or V_L domain genes by C-terminal gene fusion to the respective antibody partners such that on correct heterodimerization of these added domains the bispecific construct should be created.

(iii) Transfer of the complete individal gene constructs to an appropriate expression vector or vectors (if not performed previously) and subsequent introduction to the host organism.

(iv) Growth of the host organism and expression of the installed recombinant genes to achieve antibody production.

(v) Purification of the antibody/antibodies and any ensuing biochemical manipulation to obtain the bispecific construct.

Naturally, the details of the individual steps will

be dependant on the form of the bispecific antibody to be produced e.g $(F_v)_2.V_H/V_L$ or $F(ab)_2.V_H/V_L$, (see figs 4 and 5) whether they are to be co- or individually expressed and the host organism (e.g prokaryote or eukaryote) which is to be used.

The invention is illustrated but not limited by the following examples.

Example 1

This example illustrates the production of a bispecific antibody from two different scFv fragments expressed in Escherichia coli.

(a) Starting with the mouse hybridoma cell lines producing the monoclonal antibodies of interest, a suitable method for determining the corresponding heavy and light chain variable sequences from hybridoma in RNA is described by Orlandi et al., Proc. Natl. Acad. Sci. USA, 86, 3833-3837, 1989.

(b) Genes encoding reshaped human antibodies (comprising the necessary mouse CDR and framework residues) which correspond to the original mouse monoclonals can be produced by site directed mutagenesis (see Riechman et al.). Alternatively genes encoding the reshaped human antibodies can be assembled by gene synthesis (Jones et al.)(ref 20).

(c) These genes can be manipulated using various molecular biology techniques to form scF_v fragments as described by Bird et al., Science 242, 423-426, 1988 (ref 21) or Huston et al., Proc. Natl. Acad. Sci. USA 85,

5879-5883, 1988 (ref 22).

Complete human V_H or V_L domains (naturally paired but preferably showing no specific binding relative to the antibodies utilized) can be added by C-terminal gene fusion, one to either of the two constituent scF_v constructs.

(d) The completed genes can then be cloned into an appropriate expression vector(s) depending on whether they are to be expressed as in a two cistron system on one vector under a single promotor, expressed singularly from each carried on its own vector yet together in a co-transformed host or each individual grown in different cultures. Examples of such vectors may be obtained from the literature of Skerra et al., Science, 240 1038-1041, 1988 (ref 3), Ward et al., Nature, 341, 544-546, 1989 (ref 13), Bird et al., Science 242, 423-426, 1988 (ref 21), Hoogenboom et al., Nuc. Acid Res. 19, 4133-4137, 1991 (ref 23)., etc. Further changes to obtain vectors suited to the needs of bispecific production may be necessary.

(e) Depending on whether the bispecific formation is to be performed in vitro or in vivo and the state of the products formed it may be pertinent to incorporate an additional reduction and reoxidation of the purified antibody fragments in order to obtain the desired bispecific (Bird et al., Science 242, 423-426, 1988 (ref 21), Skerra et al., Science, 240 1038-1041, 1988 (ref3), Kostelny et al., J. Immunol. 148, 1547-1553, 1992 (ref

10).)

Similarly $F(ab)_2.V_H.V_L$ fragments can be produced in E.coli by similar means and the V_H/V_L association technique may utilized for for other antibody fragments and other dimerization purposes (see fig 5)

Example 2

This example illustrates the production of a bispecific antibody from two different Fab fragments expressed mammalian cell lines. The initial procedures are quintessencally the same as in example 1 (steps a and b)

The construction of the bispecific shown in fig.5, could be made by the same technique as was used for the $F(ab)_2$.Jun.Fos. construction as described by Kostelny et al., J. Immunol. 148, 1547-1553, 1992 (ref 10). in which the one the additional dimerization domains were fused to the first few residues of the C_H2 intron such that it would be spliced to the C_H1 of the V_H gene thus replacing the normal C_H2 and C_H3. The V_L/C_L genes are expressed from a separate vector which is co-transfected with the V_H carrying plasmid.

Again various strategies for expression and bispecific production may be attempted as have been detailed previously for E. coli expression.

REFERENCES

The following references are all hereby incorporated by reference.

1. Kohler G., & Milstein C., (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. Nature, **265**, 495-497.

2. Verhoeyen M., Milstein C., & Winter G., (1988). Reshaping human antibodies: grafting an anti-lysozyme activity. Science, **239**, 1098-1104.

3. Skera A., and Pluckthun A., (1988). Assembly of a functional immunoglobulin Fv fragment in E.coli Science, **240**, 1038-1041.

4. Better M., Chang C.P., Robinson R.R., and Horwitz A.H., (1988). E.coli secretion of an active chimaeric antibody fragment. Science, **240**, 1041-1043.

5. Winter G., & Milstein C., (1991). Man-made antibodies. Nature, **349**, 293-299.

6. Nolan O., and O'Kennedy R., (1992). Bifunctional antibodies and their potential clinical applications Int. J Clin Lab Res, **22**, 21-27.

7. Yokota T., Milenic D.E., Whitlow M., and Schlom J., (1992) Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. Cancer Res, 52, 3402-3408.

8. Skerra A., and Pluckthun A., (1991). Secretion and in vivo folding of the Fab fragment of the antibody MPC603 in E.coli influence of disulphides and cis prolines. Protein Engineering, 4, 971-979.

9. Bird R.E., and Walker B.W., (1991) Single chain antibody variable regions. TIBTECH, 9, 132-137.

10. Kostelny S.A., Cole M.S., and Tso J.Y., (1992). Formation of a bispecific antibody by the use of leucine zippers J. Immunol, 148, 1547-1553.

11. Pack P., and Pluckthun A., (1992). Miniantibodies use of amphipathic helices to produce functional, flexibly linked dimeric Fv fragments with high avidity in E.coli Biochemistry, 31, 1579-1584.

12. Carter P., Kelley R.F., Rodrigues M.L., Snedecor B., Covarrubias M., Velligan M.D., Wong W.L.T., Rowland A.M., Knotts C.E., Carver M.E., Yang M., Bourell J.H., Shepard H.M., and Henner D., (1992) High level Escherichia coli expression and production of a bivalent humanised antibody fragment.

Biotechnology, 10, 163-167.

13. Ward E.S., Gussow D.H., Griffiths A.D., Jones P.T., and Winter G., (1989) Binding activities of a repoertoire of single immunoglobulin variable domains secreted from E.coli Nature, 341, 544-546.

14. Horne C., Klein M., Polidoulis I., and Dorrington K.D., (1982). Noncovalent association between heavy and light chains of immunoglobins. III Specific interactions between VH and VL J Immunol., 129, 660-664.

15. Chaudary V.K., Batra J.K., Glallow M.G., Wilingham M.C., FitzGerald D.J., and Pastan I., (1990) A rapid method of cloning functional variable-region antibody genes in Escherichia coli as single chain immunotoxins. Proc. natl. Acad. Sci. U.S.A., 87, 1066-1070.

16. Higuchi R., (1990) Recombinant PCR. In PCR protocols: A guide to methods and applications, (Edited by Innes M.A., Gelfand D.H., Sninsky J.J., and White T.J.,) pp 177-183 Academic Press, London.

17. Ornstein L., (1964) Disc electrophesis - I Background and theory. Ann N.Y. Acad Sci, 121, 321-349.

18. Hamel P.A., Klein M.H., Smith-Gill S.J., and Dorrington K.D., (1987) Relative noncovalent association constant between immunoglobulins H andL chains is unrelated to their expression of antigen-binding activity J Immunol., **139**, 3012-3020.

19. Glockshuber R., Malia M., Pfitzinger I., and Pluckthun A., (1990). A comparison of strategies to stabilize Immunoglobulin Fv-Fragments. Biochemistry, **29**, 1362-1367.

20. Jones P.T., Dear P.H., Foote J Neuberger M.S., and Winter G., (1986) Replacing the complementary determining regions in a human antibody for those from a mouse. Nature, **321**, 522-525.

21. Bird R.E., Hardman K.D., Jacobson J.W., Johnson S., Kaufmann B.M., Lee S Lee T., Pope S H., Riordan G.S., and Whitlow M., (1988) Single chain antigen binding proteins. Science, **242**, 423-426.

22. Huston J.S., Levinson D., Mudgett-Hunter M., Tai M., Novotny J., Margolies M.N., Ridge R.J., Bruccoleri R.E., Haber E., Cera R., and Opperman H., (1988) Protein engineering of antibody binding sites: Recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in E.coli. Proc. NatR. Acad. Sci U.S.A., **85**, 5879-5883.

23. Hoogenboom H.R., Griffiths A.D., Johnson K.S., Chiswell D.J., Hudson P., and Winter G., Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains N.A.R. 4133-4137.

Claims

1. A specific binding protein having first and second binding regions which specifically recognise and bind to target entities, said binding regions being contained at least in part on respectively first and second polypeptide chains, said chains additionally incorporating respectively first and second associating domains which are capable of binding to each other, causing the first and second polypeptide chains to combine, thereby providing a single protein incorporating the binding specificities of said first and second binding regions.

2. A specific binding protein according to claim 1 wherein said first and second binding regions recognise different target entities.

3. A specific binding protein according to claim 1 or claim 2, wherein said associating domains are derived from an antibody.

4. A specific binding protein according to any one of the preceding claims wherein the associating domains are derived from a human protein (ie one which has been exposed to the human immune system).

5. A specific binding protein according to any one of the preceding claims wherein said associating domains are two

identical domains which are capable of association, even if they do not normally associate in nature.

6. A specific binding protein according to any one of claims 1 to 4, wherein said associating domains are selected from:

antibody V_H and V_L regions,

antibody C_H1 and C_L regions,

antibody $V_H.C_H1$ and $V_L.C_L$ regions.

7. A specific binding protein according to any one of the preceding claims, wherein the first and second binding regions are antibody antigen-binding domains.

8. A specific binding protein according to claim 7, wherein the antigen binding domains comprise V_H and V_L regions.

9. A specific binding protein according to claim 8, wherein the V_L and V_H regions are contained in a Fab fragment.

10. A specific binding protein according to claim 8, wherein the V_L and V_H regions are contained in a single-chain Fv fragment.

11. A specific binding protein according to claim 7, wherein either or both of the binding domains are derived

from just one of the V_H or V_L regions of an antibody.

12. A specific binding protein according to claim 11, wherein either or both of the binding domains are derived from the V_H region of an antibody.

13. DNA encoding the polypeptides of the specific binding protein of any one of the preceding claims.

14. DNA according to claim 13 contained in one or more expression vectors.

15. A host cell containing recombinant DNA according to claim 13 and capable of expression of the DNA to produce the polypeptides of the specific binding protein.

16. A host cell according to claim 13 wherein the polypeptides on expression associate to form the specific binding protein.

17. A process which comprises expressing the recombinant DNA in a host cell of claim 15 to produce the polypeptides encoded thereby, and where necessary performing post-translation manipulation or processing of the polypeptides, to produce the specific binding protein.

18. A process for producing a specific binding protein of any one of claims 1 to 12, which comprises:

(i) cloning or synthesising DNA encoding said first and second binding regions,

(ii) joining in reading frame to the DNA of said first and second binding regions DNA encoding respectively said first and second associating domains,

(iii) providing one or more expression vectors containing said fused DNA constructs of (i) and (ii),

(iv) inserting the expression vectors into a host organism, (v) culturing the host organism to express the encoded polypeptides, and

(vi) causing or allowing said first and second associating domains to combine to form the specific binding protein.

19. A pharmaceutical preparation comprising a specific binding protein according to any one of the preceeding claims.

20. A method comprising using the preparation of claims 19 to treat a human or animal patient.

21. A diagnostic reagent comprising a specific binding protein according to any one of claims 1 to 18.

22. A method comprising using the reagent of claim 21 in a diagnostic technique.

Fig.1

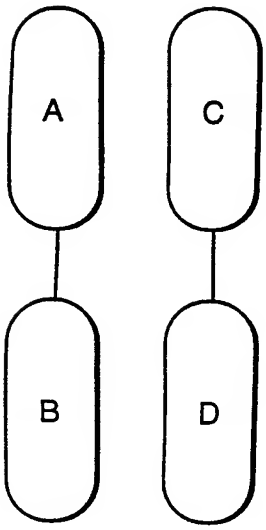


Fig.2

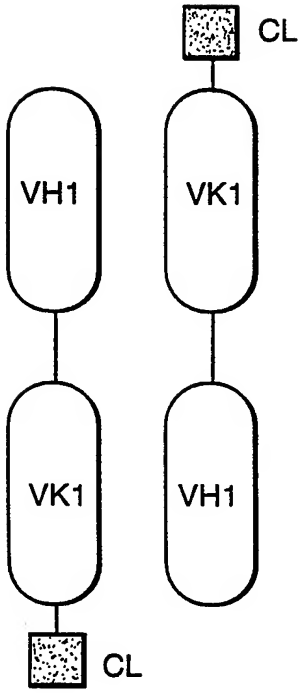


Fig.3

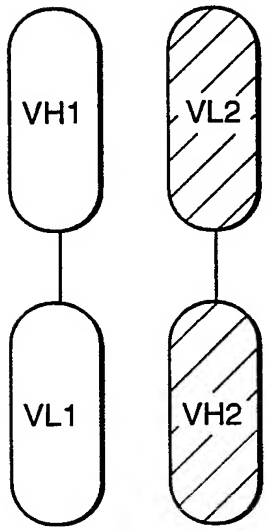


Fig.4.

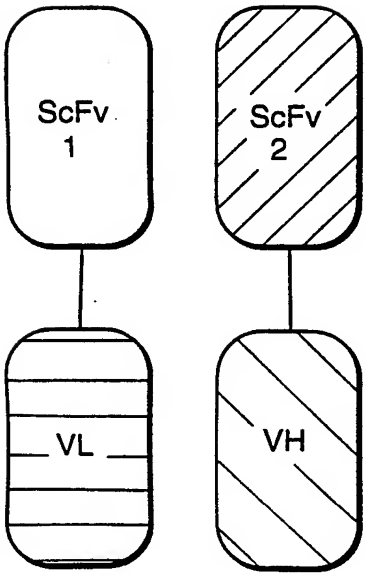


Fig.5

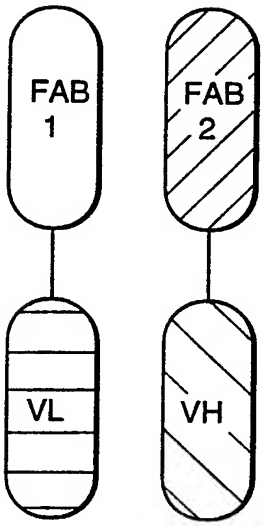


Fig.6.

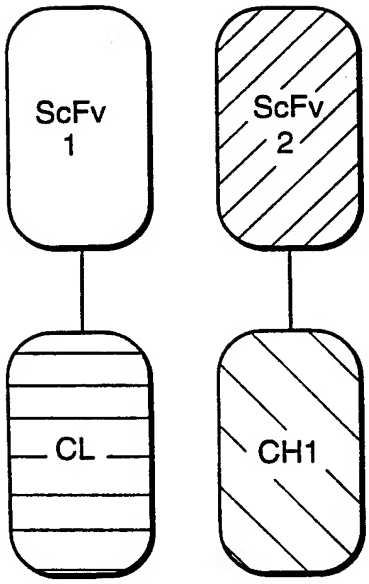


Fig.7

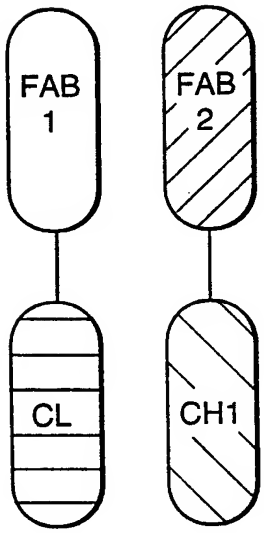


Fig.8.

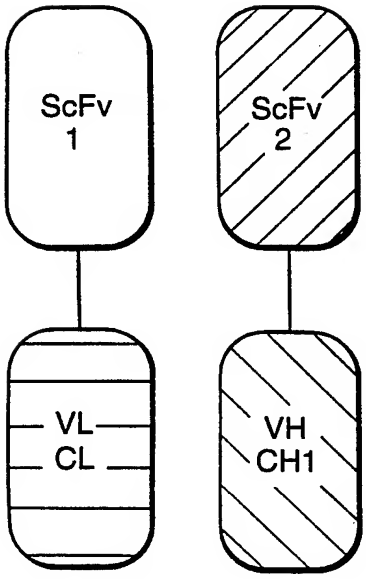


Fig.9

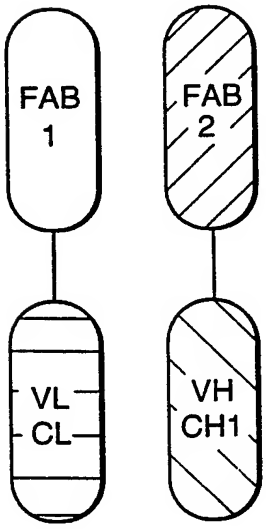


Fig.10.

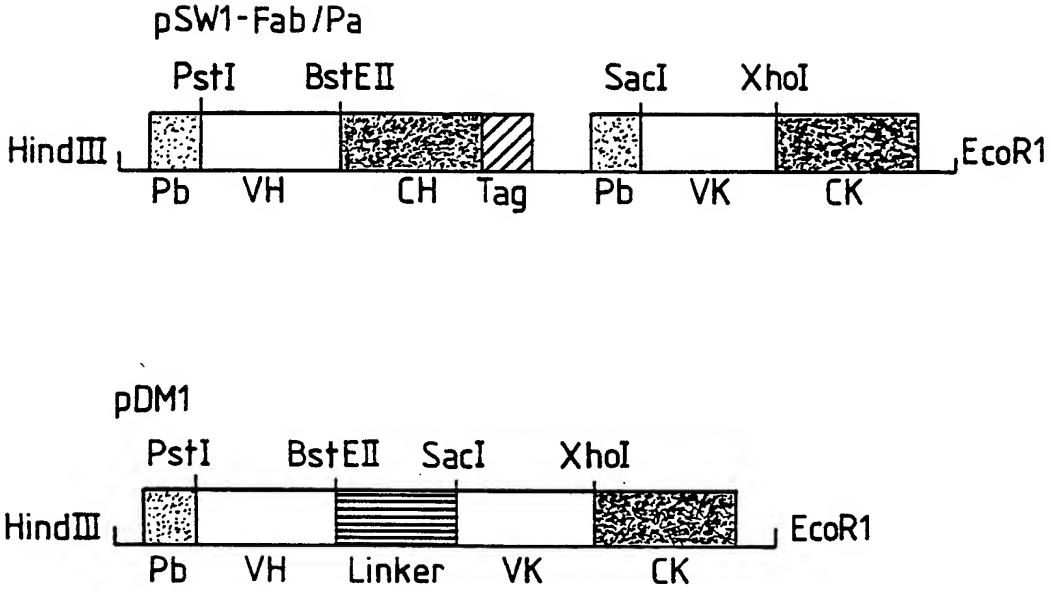

pSW1-Fab/Pa
PstI
BstEII
SacI
XhoI
HindIII
EcoR1
Pb
VH
CH
Tag
Pb
VK
CK
pDM1
PstI
BstEII
SacI
XhoI
HindIII
EcoR1
Pb
VH
Linker
VK
CK

Fig.11.

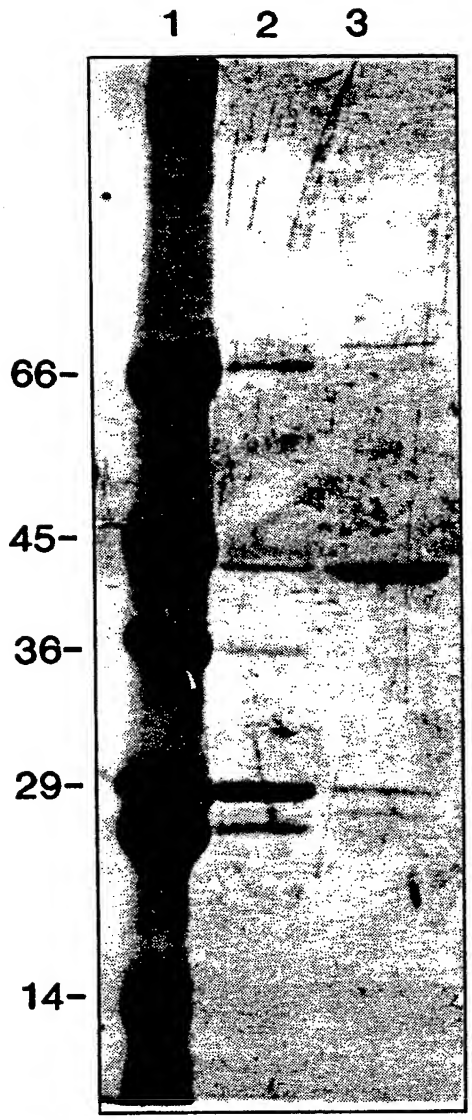


Fig.12.

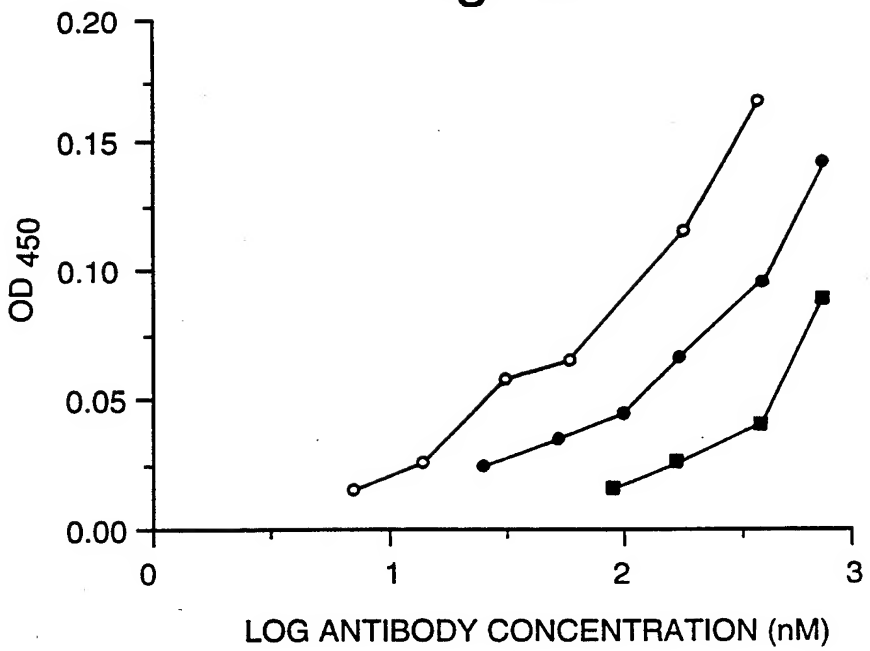

0.20
0.15
0.10
0.05
0.00
OD 450
0
1
2
3
LOG ANTIBODY CONCENTRATION (nM)

Fig.15.

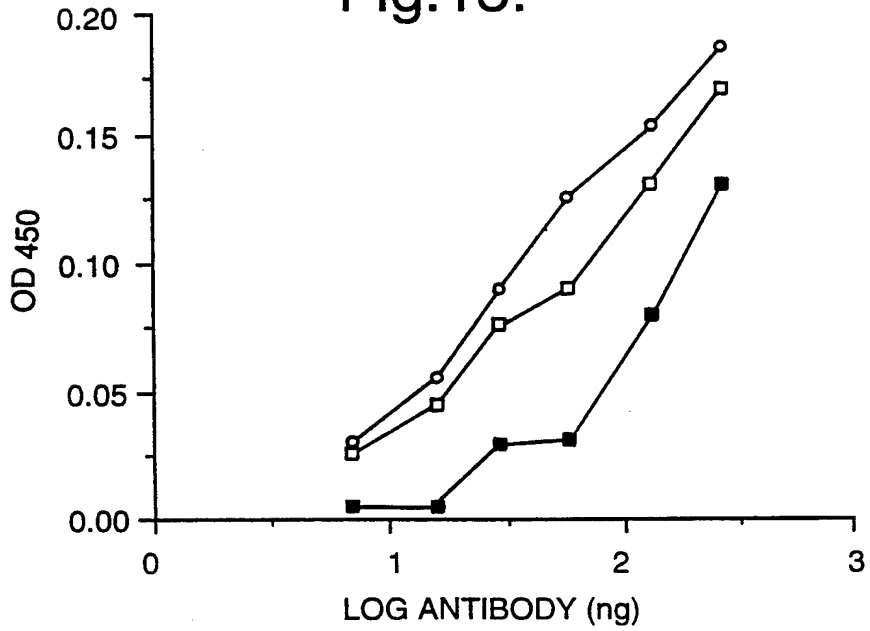

0.20
0.15
0.10
0.05
0.00
OD 450
0
1
2
3
LOG ANTIBODY (ng)

Fig.13.

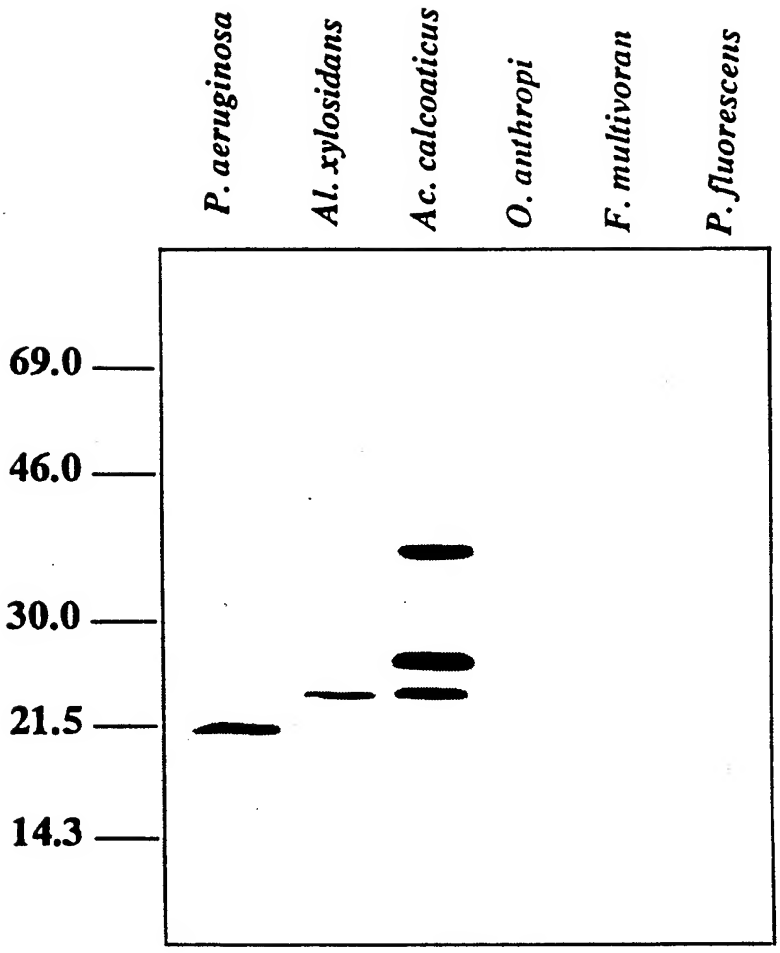

P. aeruginosa
Al. xylosidans
Ac. calcoaticus
O. anthropi
F. multivoran
P. fluorescens
69.0
46.0
30.0
21.5
14.3

Fig.14(a).

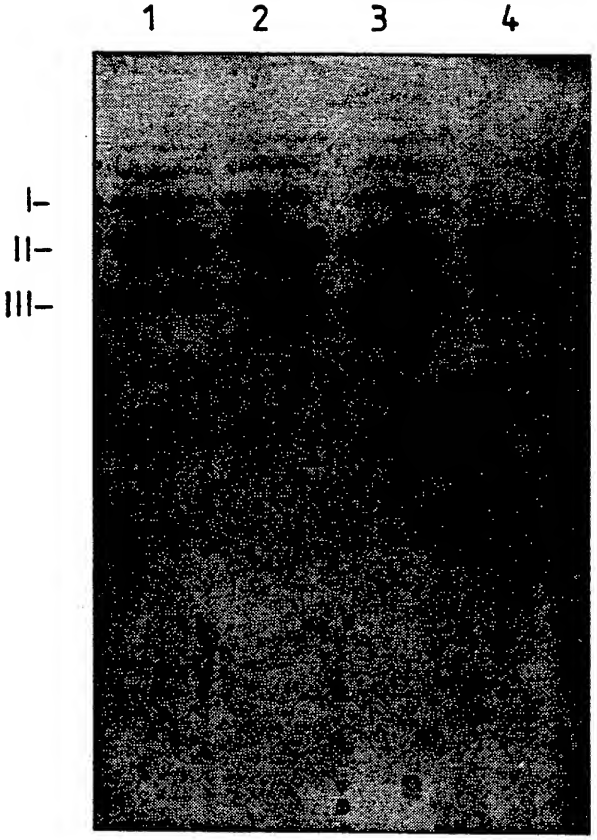
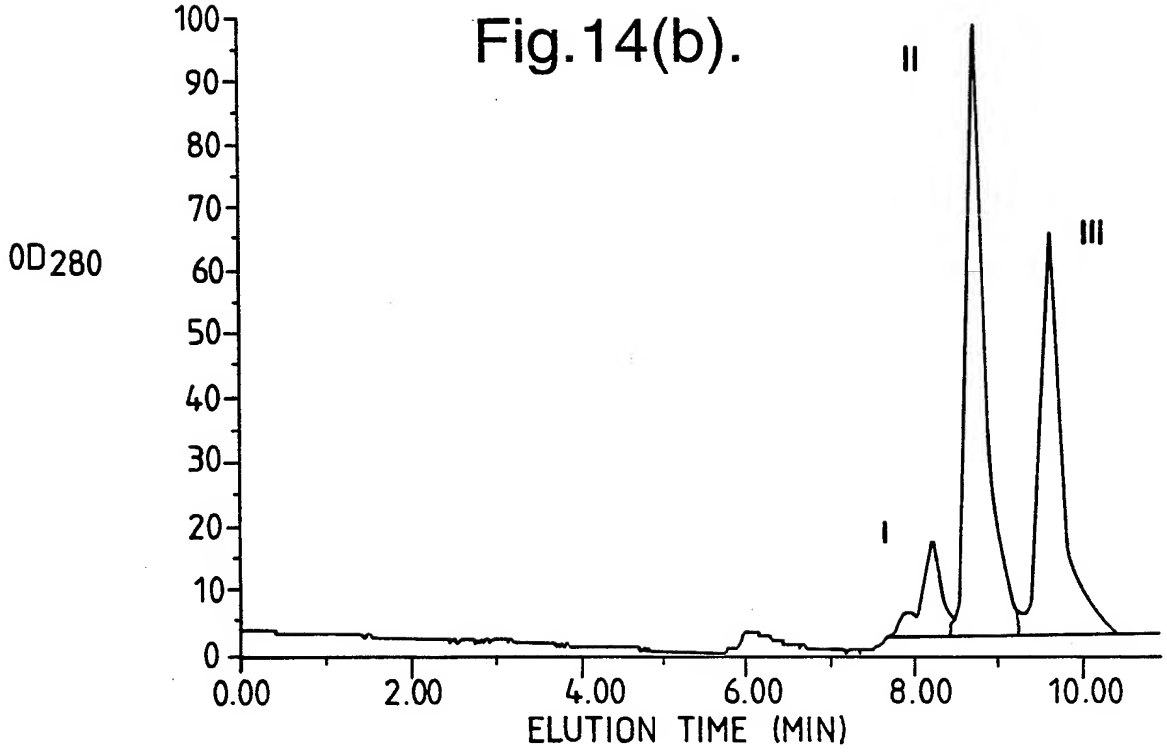


Fig.14(b).



INTERNATIONAL SEARCH REPORT

Interr ıal Application No
PCT/GB 93/02133

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/12 C12N15/13 C12N5/10 C12N1/21 C12Q1/68
A61K37/02 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N C07K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BIOCHEMISTRY. vol. 31, no. 6 , 18 February 1988 , EASTON, PA US pages 1579 - 1584 PACK, PETER ETAL.; 'Miniantibodies : use of amphipatic Helices to produce functional, flexibly linked Dimeric Fv fragments with high avidity in Esherichia Coli' see the whole document --- -/--	1-8,10, 12-16, 19-22

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
27 January 1994	21. 02. 94
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Nauche, S

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBO JOURNAL. vol. 10, no. 12 , December 1991 , EYNSHAM, OXFORD GB pages 3655 - 3659 TRAUNECKER, A. ET AL.; 'Bispecific single chain molecules (janusins) target cytotoxic lymphocytes on HIV infected cells.' see the whole document	1,2,4,6, 13-16, 19-22
X	BIOTECHNOLOGY vol. 10 , February 1992 , NEW YORK US pages 163 - 167 CARTER, P. ET AL.; 'High level Esherichia Coli expression and production of a bivalent humanized antibody fragment' see page 165, column 2, line 23 - page 166, column 2, line 22	1-8,10, 12-22
X	WO,A,91 19739 (CELLTECH LTD, GB) 26 December 1991 see the whole document	1-17, 20-22
X	WO,A,83 02285 (BOSTOON BIOMEDICAL RESEARCH INSTITUTE, US) 7 July 1983 see page 4, line 1 - page 5, line 27; claims 1-3,7,8	1-9, 19-22
A	EP,A,0 453 082 (HYBRITECH, INC., US) 23 October 1991	

2

INTERNATIONAL SEARCH REPORT

Inter ional application No.
PCT/GB93/02133

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos..
because they relate to subject matter not required to be searched by this Authority, namely:
Remark : Although claim 20 is directed to a method of treatment of the human/animal body (Rule 39.1(iv)) the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

...nformation on patent family members

Inter... nal Application No
PCT/GB 93/02133

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9119739	26-12-91	AU-A-	7983191	07-01-92
		EP-A-	0486652	27-05-92
		GB-A-	2250995	24-06-92
		JP-T-	5502039	15-04-93
WO-A-8302285	07-07-83	US-A-	4444878	24-04-84
		AU-B-	549195	16-01-86
		CA-A-	1216231	06-01-87
		DE-T-	3249285	04-10-84
		EP-A,B	0096076	21-12-83
		GB-A,B	2123030	25-01-84
EP-A-0453082	23-10-91	AU-B-	637202	20-05-93
		AU-A-	7272291	12-09-91
		US-A-	5273743	28-12-93